FEBS Letters 373 (1995) 30–34 FEBS 16084

Basal dephosphorylation controls slow gating of L-type Ca^{2+} channels in human vascular smooth muscle

Klaus Groschner[a,*], Klaus Schuhmann[a], Werner Baumgartner[b], Vassili Pastushenko[b], Hansgeorg Schindler[b], Christoph Romanin[b]

[a]*Institut für Pharmakologie und Toxikologie, Karl-Franzens-Universität Graz, Universitätsplatz 2, A-8010, Graz, Austria*
[b]*Institut für Biophysik, Universität Linz, Linz, Austria*

Received 25 June 1995; revised version received 3 August 1995

Abstract The role of cellular phosphatase activity in regulation of smooth muscle L-type Ca^{2+} channels was investigated using tautomycin, a potent and specific inhibitor of serin/threonin phosphatases type 1 and 2A. Tautomycin (1–100 nM) inhibited Ca^{2+} channel activity in smooth muscle cells isolated from human umbilical vein. Tautomycin-induced inhibition of Ca^{2+} channel activity was due to a reduction of channel availability which originated mainly from prolongation of the lifetime of unavailable states of the channel. Pretreatment of smooth muscle cells with the protein kinase inhibitor H-7 (10 μM) prevented the inhibitory effect of tautomycin. Our results suggest modulation of slow gating between available and unavailable states as a mechanism of phosphorylation-dependent down-regulation of Ca^{2+} channels in vascular smooth muscle.

Key words: L-type Ca^{2+} channel; Protein phosphatase; Cellular regulation; Vascular smooth muscle; Patch clamp

1. Introduction

Basal activity of protein phosphatases has been suggested to control the function of L-type Ca^{2+} channels by determining the lifetime of phosphorylated states of channel proteins or associated regulatory proteins [1,2,3]. Recent evidence suggests phosphorylation-dependent control of specific kinetic functions of cardiac L-type channels [2,3]. Modulatory phosphorylation of smooth muscle L-type channels differs strikingly from those of cardiac and skeletal muscle L-type channels, in that phosphorylation appears to mediate not only stimulation but also inhibition of Ca^{2+} channels in smooth muscle [4–7]. Phosphorylation-dependent inhibition of smooth muscle L-type Ca^{2+} channels has been demonstrated in studies with protein kinase activators [6,7] as well as protein phosphatase inhibitors [4,5]. Inhibitory modulation of smooth muscle Ca^{2+} channels may be based on (i) a reduction of channel availability, (ii) a reduction of open probability of available channels or (iii) suppression of both availability and open probability. For cardiac Ca^{2+} channels, phosphorylation/dephosphorylation has been suggested to affect both the slow gating between available and unavailable states of the channel as well as the fast gating between open and closed states. An increase in availability as well as in open probability of available channels was found as the basis of phosphorylation-dependent up-regulation of cardiac channels [3,8]. The changes in gating behavior associated with modulatory phosphorylation of smooth muscle L-type Ca^{2+} channels have not yet been clearly identified.

The present study was aimed at characterization of changes in the gating behavior of single Ca^{2+} channels induced by inhibition of basal dephosphorylation in vascular smooth muscle cells. Our results suggest basal activity of protein phosphatases as a major determinant of kinetic properties of L-type channels in vascular smooth muscle. We provide evidence for inhibitory modulation of slow gating between available and unavailable states as the basis of phosphorylation-dependent down-regulation of smooth muscle Ca^{2+} channels.

*Corresponding author. Fax: (43) (316) 335414.

Abbreviations: EGTA, ethylene glycol bis (β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; HEPES, *N*-2-hydroxyethylpiperazine-*N′*-ethansulfonic acid; P_0, a channel's probability of being in the open state; NP_0, mean number of open channels given by the product of N (number of active channels in a patch) times P_0.

2. Materials and methods

2.1. Cell preparation

The media of human umbilical veins was enzymatically disaggregated to obtain single smooth muscle cells as described previously [9]. The cells were resuspended and stored in a high K^+ low Cl^- extracellular solution (see below) at 4°C, and used for experimentation within 36 h.

2.2. Current measurements

Ba^{2+} currents through single Ca^{2+} channels were recorded in the membrane of intact smooth muscle cells (cell-attached mode). Cell potential was set to approximately zero by use of a high K^+ low Cl^- extracellular solution [10] which contained (mM): 110 K^+ aspartate, 20 KCl, 2 $MgCl_2$, 20 HEPES, 2 EGTA, pH was adjusted to 7.4 with *N*-methyl-D-glucamine, and pCa was adjusted according Robertson and Potter [11]. The pipette solution contained (mM): 10 $BaCl_2$, 100 NaCl, 30 TEA-Cl and 15 HEPES, pH adjusted to 7.4. Patch pipettes were fabricated from borosilicate glass (Clark Electromedical Instruments; Pangbourne UK), and had resistances of 5–10 MΩ. All experiments were performed at room temperature. Part of the experiments were performed in presence of the dihydropyridine-Ca^{2+} channel activator S(−)-BayK 8644 (0.5 μM) which was added to the pipette solution. Other drugs were applied via a perfusion system which allowed complete exchange of the bath solution within approximately 15 s.

Voltage clamp and current amplification was performed with a List EPC/7 patch-clamp amplifier (List, Darmstadt, Germany). For data analysis, current records were filtered at 1 kHz (−3 dB) and digitized at a rate of 5 kHz. For display, current records were filtered at 500 Hz (−3 dB) and digitized at a rate of 2 kHz. Voltage protocols were controlled with pClamp software (Axon Instruments, Foster City, CA, USA). Idealization of current records was performed with pClamp software, as well as with a custom made level detection software based on optimization of likelihood of transitions [12]. The availability of channels to open upon depolarization was expressed as the proportion of sweeps which showed at least one opening according to the applied criteria of level detection (Student's *t*-test) [12]. Stability of gating behavior was tested in control experiments which demonstrated stability of open time distributions, unitary current amplitude and mean number of open channels in cell-attached patches of four cells in the absence of

SSDI 0014-5793(95)01012-2

pharmacological interventions over a period of 20 min. Averaged data are given as mean ± S.E.M. from the indicated number of experiments. Statistical analysis was performed using Student's *t*-test for paired values and differences were considered statistically significant at $P < 0.05$.

2.3. Materials

Collagenase, type CLS II and soybean trypsin inhibitor type were obtained from Worthington Biochemical Corporation (Freehold, NJ, USA); dispase type II was from Boehringer (Mannheim, Germany); fatty acid-free bovine serum albumin from Behring (Marburg, Germany); Hanks balanced salt solution was from Sera Lab Ltd. (Sussex, GB); S(−)-BayK 8644 and H-7 was from Research Biochemicals Incorporated (Natic, MA, USA); tautomycin was purchased from Calbiochem (San Diego, CA, USA), and all other chemicals from Sigma Chemical Co. (Deisenhofen, Germany).

3. Results

3.1. Tautomycin inhibits L-type Ca^{2+} channels in human vascular smooth muscle

L-type channels were recorded in the cell-attached configuration using 10 mM Ba^{2+} as charge carrier. Under these conditions, channels exhibited a unitary conductance of approximately 13 pS (12.9 ± 2, $n = 5$) and a threshold of activation above −40 mV. The protein phosphatase inhibitor tautomycin substantially inhibited Ca^{2+} channel activity at concentrations as low as 10 nM. Fig. 1 illustrates inhibitory effects induced by 10 nM and 100 nM tautomycin on channel activity recorded in the presence of 0.5 μM BayK 8644. Fig. 1A shows a diary plot of channel activity given as mean number of open channels (NP_0) during individual depolarizing voltage steps from a holding potential of −70 mV to a test potential of −20 mV. Mean NP_0 (calculated for 5–10 min intervals) was clearly reduced from 0.2 to 0.12 and further down to 0.05 in the presence of 10 nM and 100 nM tautomycin, respectively. At 100 nM tautomycin, a striking change in the activity pattern was observed. Extremely long periods without channel activity alternated with active periods in which NP_0 of individual sweeps was almost as high as in control. Fig. 1B shows representative current traces recorded in the absence and presence of 100 nM tautomycin. Corresponding ensemble average currents derived from 200 depolarizing voltage steps are depicted in Fig. 1C. Single channel amplitude remained unchanged in the presence of tautomycin, and open time distribution was only moderately affected. In most cells, tautomycin induced the occurrence of a relatively small proportion of extremely long-lasting channel openings. This effect varied considerably regarding the apparent time constant and proportion of the tautomycin-induced long-lasting openings, and was not further analyzed in detail. The prominent effect of tautomycin was apparently an increase in the proportion of blank sweeps which resulted in a clear reduction of the current average (Fig. 1C).

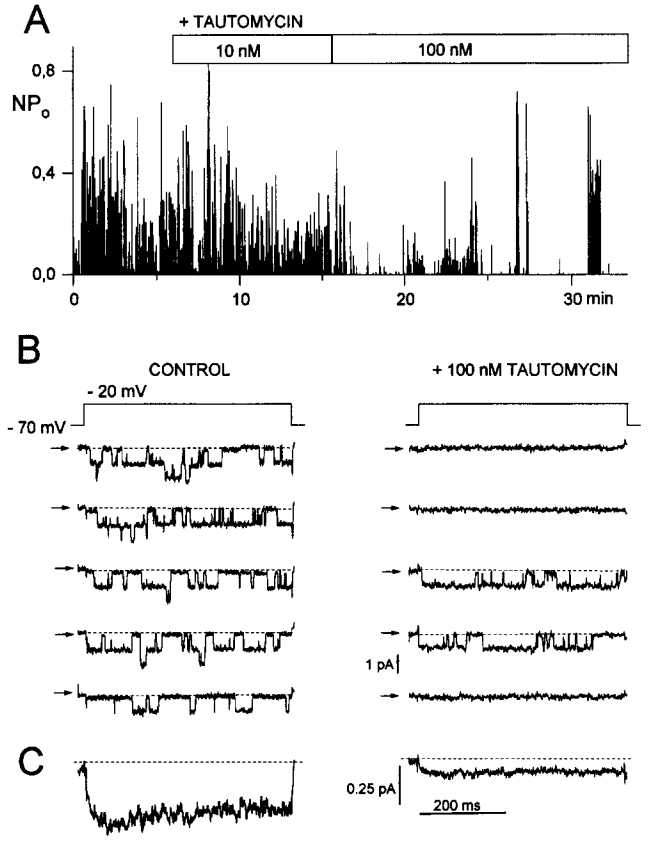

Fig. 1. Inhibition of L-Type Ca^{2+} channels by tautomycin. (A) Diary of channel activity (given as NP_0) calculated from individual depolarizing voltage pulses (holding potential −70 mV, test potential −20 mV, duration 500 ms, rate 0.66 Hz). Exposure of the cell to 10 nM and 100 nM tautomycin is indicated. (B) Individual current responses. Records are filtered at 500 Hz and digitized at 2 kHz. Zero current levels are indicated by arrows or dashed lines, respectively. (C) Ensemble average currents obtained from 200 depolarizations in the absence (control) and in the presence of 100 nM tautomycin. BayK 8644 (0.5 μM) was present in the pipette solution.

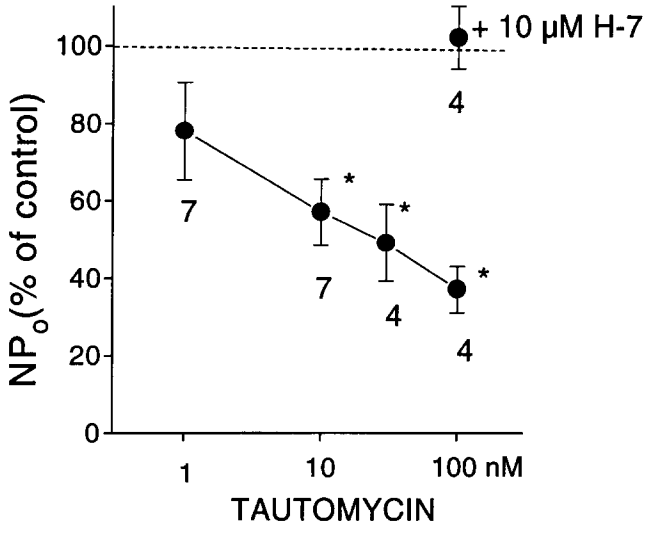

Fig. 2. Concentration-dependent inhibition of L-type Ca^{2+} channels by tautomycin and prevention of its inhibitory action by H-7. Mean values of NP_0 (% of control) ± S.E.M. were calculated from the indicated number of experiments. Statistically significant differences ($P < 0.05$ versus control) are indicated (*). BayK 8644 (0.5 μM) was present in the pipette solution.

Fig. 2 shows the concentration–response relationship of the inhibitory action of tautomycin. The threshold of its inhibitory action was as low as 1 nM. At 100 nM tautomycin, NP_0 was inhibited down to 36 ± 8% ($n = 4$) of control. To test whether the tautomycin-induced inhibition of Ca^{2+} channels is indeed due to inhibition of basal dephosphorylation, we performed a set of experiments in which the inhibitory effect of 100 nM tautomycin was tested in the presence of 10 μM H-7, a non

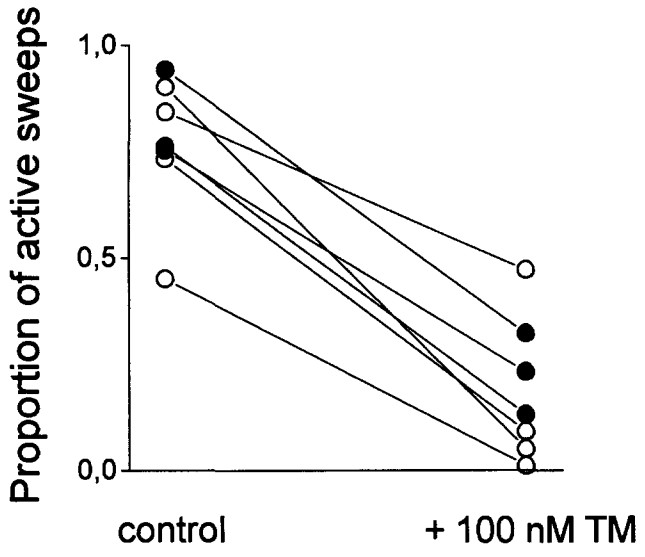

Fig. 3. Tautomycin-induced reduction of the proportion of active sweeps. The fraction of active sweeps was calculated for periods of 3–5 min in the absence and presence of 100 nM tautomycin and shown for individual experiments. Filled symbols represent experiments in which BayK 8644 (0.5 μM) was present in the pipette solution.

specific inhibitor of protein kinases. Based on the concept of reversible phosphorylation/dephosphorylation, stabilization of phosphorylated states of the channel or regulatory proteins by phosphatase inhibitors are expected to depend on the rate of basal phosphorylation. Thus, inhibition of protein kinases is expected to suppress the effects of phosphatase inhibition. Preincubation of cells with 10 μM H-7 indeed completely antagonized the effects of 100 nM tautomycin. No change in NP_0 was induced by 100 nM tautomycin in the presence of H-7 ($n = 4$) as illustrated in Fig. 2.

3.2. *Tautomycin-induced inhibition of smooth muscle Ca^{2+} channels is due to prolongation of the lifetime of unavailable states of the channel*

Tautomycin (100 nM) consistently diminished the fraction of depolarizing pulses which elicited channel openings (active sweeps), indicating an inhibitory effect on channel availability. This result is illustrated in Fig. 3. To test whether modulation of channels by BayK 8644 is of relevance regarding the observed tautomycin effects on gating kinetics, the dihydropyridine activator was omitted in a set of experiments. The results obtained with four patches which exhibited a sufficiently high channel activity for evaluation of inhibitory effects are included in Fig. 3. Irrespective of the presence or absence of the dihydropyridine activator, tautomycin reduced the proportion of active sweeps in each individual experiment. In one of the experiments performed in the absence of BayK 8644, only one single Ca^{2+} channel was detected, and its activity could be recorded for more than 40 min. This situation allowed for a detailed analysis of the slow gating behavior. The channel was found available in about 50% of the depolarizing pulses. Fig. 4A shows a diary-plot of the channel's P_0. According to the idea of slow gating of Ca^{2+} channels between available and unavailable states [3,8], the occurrence of active and blank sweeps was clustered. Consecutive current records obtained in the absence and presence of tautomycin are illustrated in Fig. 4B. Tautomycin (100 nM) enhanced the proportion of blank sweeps (marked with B in Fig. 4B), while open probability of the channel during active sweeps remained almost unchanged. Tautomycin-induced suppression of channel availability was clearly borne out by a prolongation of uninterrupted clusters of blank sweeps (blank runs). Distribution of the lifetime of active and blank runs are shown in Fig. 3C. Data were best fitted with double exponential functions. Tautomycin exerted a modest effect on the duration of active runs but markedly increased the mean lifetime of blank runs (given as number of depolarizing pulses, i.e. sweeps). Time constants derived from active sweep histograms decreased from 0.8 and 4.9 (number of sweeps) in control to 0.3 and 2.8 in the presence of tautomycin, while time constants derived from blank sweep histograms increased from control values of 0.4 and 2.7 to 3.3 and 19,1 in the presence of tautomycin. Fast gating of the channel was barely affected by tautomycin as evident from analysis of open probability and open time distribution. Open probability of the available channel was 0.14 in the absence and 0.11 in the presence of tautomycin. Biexponential fit of open time distributions yielded time constants of 0.4 ms for short and 5.9 ms for long openings in control, and 0.6 ms and 7.3 ms in the presence of tautomycin. The proportion of long lasting openings was 39% in control and 42% in the presence of tautomycin.

4. Discussion

With the present study, we provide evidence for phosphorylation-dependent inhibition of smooth muscle L-type Ca^{2+} channel activity, based on modulation of slow gating properties of the channel. Inhibition of basal dephosphorylation clearly promoted unavailable states of the Ca^{2+} channel. Our results suggest that the slow gating between available and unavailable states of the channel is in large part determined by the activity of protein phosphatases in the smooth muscle cell.

We used tautomycin to suppress basal dephosphorylation in human vascular smooth muscle cells. Tautomycin is a bacterial toxin which is known as a highly specific inhibitor of serine/threonine phosphoprotein phosphatases [13]. Tautomycin markedly inhibited mean Ca^{2+} channel activity in human vascular smooth muscle. This finding is in accordance with the concept of smooth muscle L-type Ca^{2+} channels being subject to phosphorylation-dependent down-regulation [4–7]. Inhibitory phoshorylation of smooth muscle Ca^{2+} channels has been suggested to involve cyclic nucleotide-dependent protein kinases [6] and/or specific isoforms of protein kinase C [7]. These mechanisms of cellular regulation are considered as reversible phosphorylation/dephosphorylation processes. Thus inhibition of protein phosphatases may well promote low activity of smooth muscle Ca^{2+} channels. Our results are in line with previous reports demonstrating inhibition of whole-cell Ca^{2+} currents in smooth muscle by phosphatase inhibitors [4,5]. Tautomycin-induced inhibition of Ca^{2+} channel activity was due to a reduction of the channels' ability to open upon depolarization, i.e. the channels' availability. We have recently reported on a dual regulation of human smooth muscle Ca^{2+} channels by activation of protein kinase C [7], and demonstrated that protein kinase C-mediated inhibition is associated with reduced channel availability. Similarly the inhibitory effect of tautomycin was found to be based predominantly on a reduction of channel

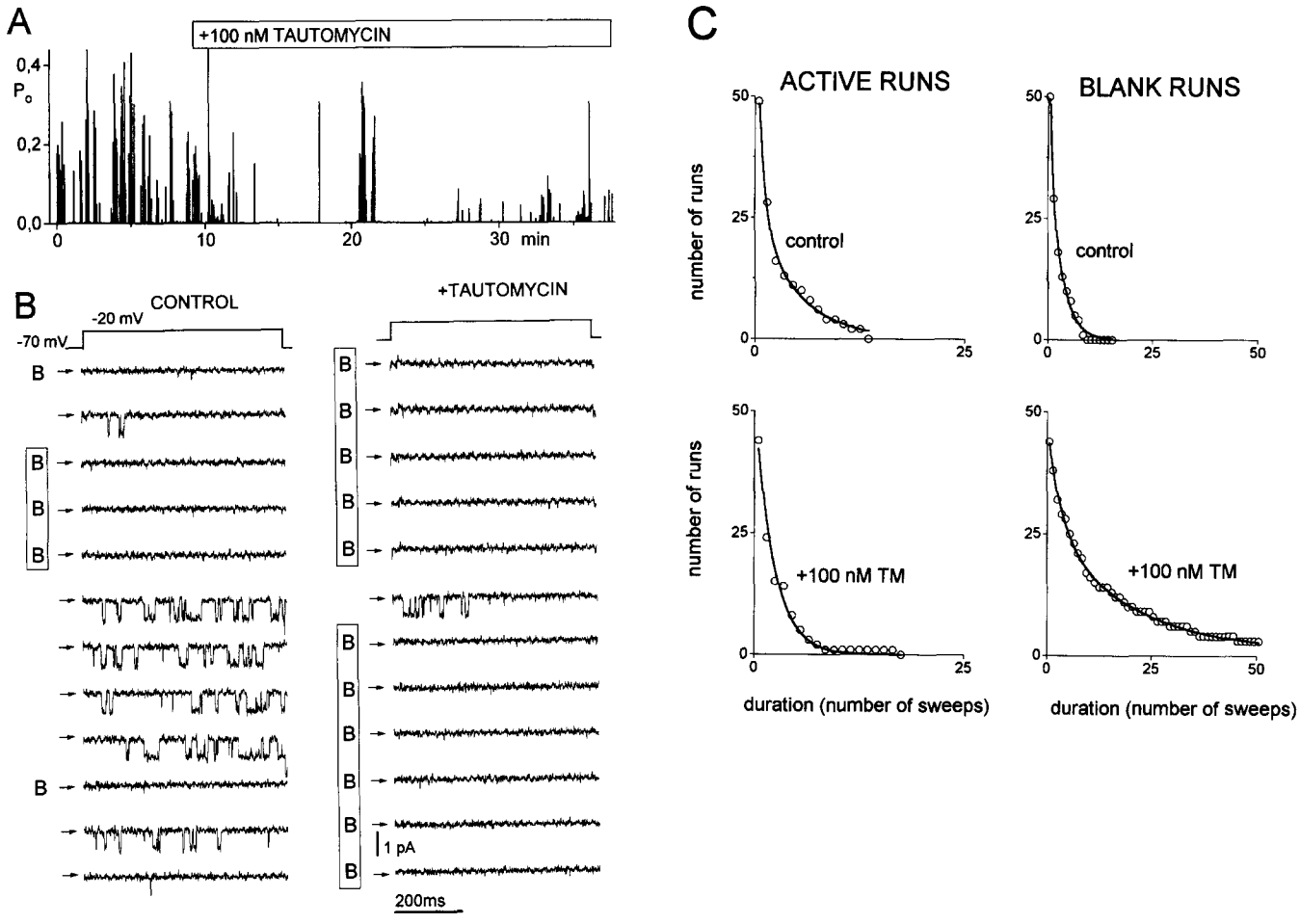

Fig. 4. Tautomycin-induced changes in slow gating behavior of a single Ca^{2+} channel. (A) Diary of a channel's P_0 calculated from individual depolarizing voltage pulses (holding potential −70 mV, test potential −20 mV, duration 500 ms, rate 0.66 Hz). (B) Consecutive current traces recorded in the absence and presence of 100 nM tautomycin. Closed state is indicated by arrows or dashed lines, respectively. Blank sweeps (B) are marked. Records are filtered at 500 Hz and digitized at 2 kHz. (C) Sweep histograms, plotting the number of runs (uninterrupted clusters of active or blank sweeps) against the duration of runs (number of sweeps) are shown for control and the presence of 100 nM tautomycin. No BayK 8644 was present in the pipette solution.

availability. Thus, besides cyclic nucleotide-dependent protein kinases, isoforms of protein kinase C may be involved in the observed down-regulation of smooth muscle Ca^{2+} channels. Considering the concept of reversible phosphorylation/dephosphorylation as the basis of channel regulation, the effect of a phosphatase inhibitor should critically depend on the level of basal protein kinase activity. In accordance with this hypothesis, H-7, an inhibitor of protein kinase C and cyclic nucleotide-dependent protein kinases, prevented the effects of tautomycin.

Analysis of slow gating of a single Ca^{2+} channel in the absence of BayK 8644 yielded blank sweep histograms which were best described by the sum of two exponential functions indicating the existence of at least two unavailable states of the channel. A similar result has been reported for cardiac channels [3]. The phosphatase inhibitor tautomycin markedly prolonged the lifetime of unavailable states of the channel. This result suggests protein dephosphorylation as a mechanism involved in the gating transition from unavailable to available states. It is tempting to interpret the effects of tautomycin on channel kinetics in terms of distinct phosphorylated states of the Ca^{2+} channel which are stabilized by inhibition of basal dephosphorylation. This idea is in line with the finding that serine/threonine protein phosphatases selectively dephosphorylate different sites on the α_1 and β subunit of the Ca^{2+} channel [14]. Since coupling between voltage sensing and pore opening in the α_1 subunit was found to be determined by its interaction with the β subunit [15], not only the pore forming α_1 subunit but also the β subunit of the channel complex may well serve as a target of modulatory phosphorylation. The involvement of phosphorylation sites on regulatory proteins beside the channel complex, however, cannot be ruled out at present. Nonetheless, our results provide clear evidence for phosphorylation-dependent down-regulation of smooth muscle Ca^{2+} channels via suppression of channel availability.

Our study is the first demonstration of smooth muscle Ca^{2+} channel slow gating being controlled via basal phosphatase activity. This mechanism may be of particular physiologic and pathophysiologic relevance, since changes in cellular phosphatase activity have been suggested as key events in hormonal control of smooth muscle functions [16]. The presented results

suggest basal deposphorylation as a major determinant of smooth muscle Ca^{2+} channel kinetics and consequently of voltage-dependent Ca^{2+} entry into smooth muscle cells.

Acknowledgements: This work was supported by the Fonds zur Förderung der Wissenschaftlichen Forschung (Projects S6605, S6606 and S6610). We wish to thank Mrs. R. Schmidt and Mr. M. Ribitsch for excellent technical assistance.

References

[1] Hescheler, J., Kameyama, M., Trautwein, W., Mieskes, G. and Söling, H. (1987) Eur. J. Biochem. 165, 261–266.
[2] Ono, K. and Fozzard, H.A. (1993) J. Physiol. 470, 73–84.
[3] Herzig, S., Patil, P., Neumann, J., Staschen, C.M. and Yue, D.T. (1993) Biophysical J. 65, 1599–1612.
[4] Obara, K. and Yabu, H. (1993) Am. J. Physiol. 264, C296–C301.
[5] Ward, S.M., Vogalis, F., Blonfield, D.P., Ozaki, H., Fusetani, H., Uemura, D., Publicover, N.G. and Sanders K.M. (1991) Am. J. Physiol. 261, C64–C70.
[6] Ishikawa, T., Hume, J.R. and Keef, K.D. (1993) Circ. Res. 73, 1128–1137.
[7] Schuhmann, K. and Groschner, K. (1994) FEBS Lett. 341, 208–212.
[8] Ochi, R. and Kawashima, Y. (1990) J. Physiol. 424, 178–204.
[9] Groschner, K., Graier, W.F. and Kukovetz, W.R. (1994) Circ Res. 75, 304–314.
[10] Romanin, C., Grosswagen, P. and Schindler, H. (1991) Pflügers Arch. 418, 86–92.
[11] Robertson, S.P. and Potter, J.D. (1984) Methods Pharmacol. 5, 63–75.
[12] Pastushenko, V.P.H. and Schindler, H. (1993) Acta Pharm. 43, 7–13.
[13] MacKintosh, C. and Klumpp, S. (1990) FEBS Lett. 277, 137–140.
[14] Lai, Y. Peterson, B.Z. and Catterall, W.A. (1993) J. Neurochem. 61, 1333–1339.
[15] Neely, A. Wei, X., Olcese, R., Birnbaumer, L. and Stefani, E. (1993) Science 262, 575–578.
[16] Somlyo, A.P. and Somlyo, A.V. (1994) Nature 372, 231–236.